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(54) Title: NETRIN RECEPTORS

(57) Abstract

The invention provides methods and compositions relating to vertebrate UNC-5 proteins which function as receptor proteins for netrins, a family of cell guidance proteins. The proteins may be produced recombinantly from transformed host cells from the disclosed vertebrate UNC-5 encoding nucleic acid or purified from human cells. The invention provides specific hybridization probes and primers capable of specifically hybridizing with the disclosed vertebrate *unc-5* gene, vertebrate UNC-5-specific binding agents such as specific antibodies, and methods of making and using the subject compositions in diagnosis, therapy and in the biopharmaceutical industry.

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Netrin Receptors

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INTRODUCTION**Field of the Invention**

10 The field of this invention is proteins which regulate vertebrate cell guidance.

Background

In the developing nervous system, migrating cells and axons are guided to their targets by cues in the extracellular environment. The netrins are a family of phylogenetically-conserved guidance cues that can function as diffusible attractants and repellents for different 15 classes of cells and axons¹⁻¹⁰. Recent studies in vertebrates, insects and nematodes have implicated members of the DCC subfamily of the immunoglobulin (Ig) superfamily as receptors involved in migrations toward netrin sources^{6, 11-13}. The mechanisms that direct migrations away from netrin sources (presumed repulsions) are less well understood. In *Caenorhabditis elegans*, loss of *unc-5* (which encodes the transmembrane protein UNC-5¹⁴) 20 function causes defects in these migrations^{15, 16}, and ectopic expression of *unc-5* in some neurons can redirect their axons away from a netrin source¹⁷. However, the relationship between UNC-5 and the netrins has not been defined. We disclose herein vertebrate homologues of the *C. elegans* UNC-5, which define a novel subfamily of the Ig superfamily, and whose mRNAs show prominent expression in various classes of differentiating neurons 25 and we disclose that these vertebrate UNC-5 homologues are vertebrate netrin-binding proteins.

SUMMARY OF THE INVENTION

The invention provides methods and compositions relating to vertebrate UNC-5 30 proteins, related nucleic acids, and protein domains thereof having vertebrate UNC-5-specific activity. The proteins may be produced recombinantly from transfected host cells from the

subject vertebrate UNC-5 encoding nucleic acids or purified from vertebrate cells. The invention provides isolated vertebrate *unc-5* hybridization probes and primers capable of specifically hybridizing with the disclosed vertebrate *unc-5* genes, vertebrate UNC-5-specific binding agents such as specific antibodies, and methods of making and using the subject compositions in diagnosis (e.g. genetic hybridization screens for vertebrate *unc-5* transcripts),
5 therapy (e.g. gene therapy to modulate vertebrate *unc-5* gene expression) and in the biopharmaceutical industry (e.g. as immunogens, reagents for modulating cell guidance, reagents for screening chemical libraries for lead pharmacological agents, etc.).

DETAILED DESCRIPTION OF THE INVENTION

10 The nucleotide sequences of natural *unc5h-1* cDNAs from rat and human are shown as SEQ ID NOS:1 and 2, respectively; and the conceptual translates are shown as SEQ ID NOS: 5 and 6, respectively. The nucleotide sequences of natural *unc5h-2* cDNAs from rat and human are shown as SEQ ID NOS:3 and 4, respectively; and the conceptual translates are shown as SEQ ID NOS:7 and 8, respectively. The vertebrate UNC-5 proteins of the
15 invention include incomplete translates of SEQ ID NOS:1, 2, 3 and 4 and deletion mutants of SEQ ID NOS:5, 6, 7 and 8, which translates and deletion mutants have vertebrate UNC-5-specific amino acid sequence and assay-discriminable vertebrate UNC-5-specific binding specificity or function. Such active vertebrate UNC-5 deletion mutants, vertebrate UNC-5 peptides or protein domains comprise at least about 8, preferably at least about 12, more
20 preferably at least about 24 consecutive residues of SEQ ID NO:5, 6, 7 or 8. For examples, vertebrate UNC-5 protein domains identified below are shown to provide protein-binding domains which are identified in and find use, *inter alia*, in solid-phase binding assays as described below.

25 Vertebrate UNC-5-specific activity or function may be determined by convenient *in vitro*, cell-based, or *in vivo* assays: e.g. *in vitro* binding assays, cell culture assays, in animals (e.g. gene therapy, transgenics, etc.), etc. Binding assays encompass any assay where the molecular interaction of a vertebrate UNC-5 protein with a binding target is evaluated. The binding target may be a natural extracellular binding target such as a netrin protein, or other regulator that directly modulates vertebrate UNC-5 activity or its localization; or non-natural
30 binding target such a specific immune protein such as an antibody, or an vertebrate UNC-5 specific agent such as those identified in screening assays such as described below.

Vertebrate UNC-5-binding specificity may assayed by binding equilibrium constants (usually at least about 10^7 M^{-1} , preferably at least about 10^8 M^{-1} , more preferably at least about 10^9 M^{-1}), by the ability of the subject protein to function as negative mutants in vertebrate UNC-5-expressing cells, to elicit vertebrate UNC-5 specific antibody in a heterologous mammalian host (e.g a rodent or rabbit), etc. In any event, the vertebrate UNC-5 binding specificity of the subject vertebrate UNC-5 proteins necessarily distinguishes *C. elegans* UNC-5.

5 The claimed vertebrate UNC-5 proteins are isolated or pure: an "isolated" protein is unaccompanied by at least some of the material with which it is associated in its natural state, preferably constituting at least about 0.5%, and more preferably at least about 5% by weight of the total protein in a given sample and a pure protein constitutes at least about 90%, and
10 preferably at least about 99% by weight of the total protein in a given sample. The vertebrate UNC-5 proteins and protein domains may be synthesized, produced by recombinant technology, or purified from mammalian, preferably human cells. A wide variety of molecular and biochemical methods are available for biochemical synthesis, molecular expression and purification of the subject compositions, see e.g. Molecular Cloning, A
15 Laboratory Manual (Sambrook, *et al.* Cold Spring Harbor Laboratory), Current Protocols in Molecular Biology (Eds. Ausubel, *et al.*, Greene Publ. Assoc., Wiley-Interscience, NY) or that are otherwise known in the art.

20 The invention provides natural and non-natural vertebrate UNC-5-specific binding agents, methods of identifying and making such agents, and their use in diagnosis, therapy and pharmaceutical development. For example, vertebrate UNC-5-specific agents are useful in a variety of diagnostic and therapeutic applications. Vertebrate UNC-5-specific binding agents include vertebrate UNC-5-specific ligands, such as netrins, and somatically recombined protein receptors like specific antibodies or T-cell antigen receptors (see, e.g Harlow and Lane (1988) Antibodies, A Laboratory Manual, Cold Spring Harbor Laboratory)
25 and other natural binding agents identified with assays such as one-, two- and three-hybrid screens, non-natural binding agents identified in screens of chemical libraries such as described below, etc. For diagnostic uses, the binding agents are frequently labeled, such as with fluorescent, radioactive, chemiluminescent, or other easily detectable molecules, either conjugated directly to the binding agent or conjugated to a probe specific for the binding
30 agent. Agents of particular interest modulate vertebrate UNC-5 function, e.g. vertebrate UNC-5-dependent cell guidance; for example, isolated cells, whole tissues, or individuals

may be treated with a vertebrate UNC-5 binding agent to activate, inhibit, or alter vertebrate UNC-5-dependent cell guidance or function.

The invention provides UNC-5 related nucleic acids, which find a wide variety of applications including use as translatable transcripts, hybridization probes, PCR primers, diagnostic nucleic acids, etc.; use in detecting the presence of *unc-5* genes and gene transcripts and in detecting or amplifying nucleic acids encoding additional *unc-5* homologs and UNC-5 structural analogs. The subject nucleic acids are of synthetic/non-natural sequences and/or are isolated, i.e. unaccompanied by at least some of the material with which it is associated in its natural state, preferably constituting at least about 0.5%, preferably at least about 5% by weight of total nucleic acid present in a given fraction, and usually 5 recombinant, meaning they comprise a non-natural sequence or a natural sequence joined to nucleotide(s) other than that which it is joined to on a natural chromosome. Nucleic acids 10 comprising the nucleotide sequence of SEQ ID NO:1, 2, 3 or 4 or fragments thereof, contain such sequence or fragment at a terminus, immediately flanked by a sequence other than that which it is joined to on a natural chromosome, or flanked by a native flanking region fewer 15 than 10 kb, preferably fewer than 2 kb, which is at a terminus or is immediately flanked by a sequence other than that which it is joined to on a natural chromosome. While the nucleic acids are usually RNA or DNA, it is often advantageous to use nucleic acids comprising other bases or nucleotide analogs to provide modified stability, etc.

The amino acid sequences of the disclosed vertebrate UNC-5 proteins are used to 20 back-translate vertebrate UNC-5 protein-encoding nucleic acids optimized for selected expression systems (Holler et al. (1993) Gene 136, 323-328; Martin et al. (1995) Gene 154, 150-166) or used to generate degenerate oligonucleotide primers and probes for use in the isolation of natural vertebrate UNC-5-encoding nucleic acid sequences ("GCG" software, Genetics Computer Group, Inc, Madison WI). vertebrate UNC-5-encoding nucleic acids used 25 in vertebrate UNC-5-expression vectors and incorporated into recombinant host cells, e.g. for expression and screening, transgenic animals, e.g. for functional studies such as the efficacy of candidate drugs for disease associated with vertebrate UNC-5-modulated transcription, etc.

The invention also provides nucleic acid hybridization probes and replication / 30 amplification primers having a vertebrate UNC-5 cDNA specific sequence contained in SEQ ID NO:1, 2, 3 or 4 and sufficient to effect specific hybridization thereto (i.e. specifically hybridize with the corresponding SEQ ID NO:1, 2, 3 or 4 in the presence of *C. elegans unc-5*

cDNA). Such primers or probes are at least 12, preferably at least 24, more preferably at least 36 and most preferably at least 96 bases in length. Demonstrating specific hybridization generally requires stringent conditions, for example, hybridizing in a buffer comprising 30% formamide in 5 x SSPE (0.18 M NaCl, 0.01 M NaPO₄, pH7.7, 0.001 M EDTA) buffer at a temperature of 42°C and remaining bound when subject to washing at 42°C with 0.2 x SSPE; 5 preferably hybridizing in a buffer comprising 50% formamide in 5 x SSPE buffer at a temperature of 42°C and remaining bound when subject to washing at 42°C with 0.2 x SSPE buffer at 42°C. vertebrate UNC-5 cDNA homologs can also be distinguished from other protein using alignment algorithms, such as BLASTX (Altschul *et al.* (1990) Basic Local Alignment Search Tool, J Mol Biol 215, 403-410).

10 Vertebrate *unc-5* hybridization probes find use in identifying wild-type and mutant vertebrate *unc-5* alleles in clinical and laboratory samples. Mutant alleles are used to generate allele-specific oligonucleotide (ASO) probes for high-throughput clinical diagnoses. Therapeutic vertebrate UNC-5 nucleic acids are used to modulate cellular expression or intracellular concentration or availability of active vertebrate UNC-5. For example, 15 vertebrate UNC-5 nucleic acids are also used to modulate cellular expression or intracellular concentration or availability of active vertebrate UNC-5 protein. Vertebrate UNC-5 inhibitory nucleic acids are typically antisense: single-stranded sequences comprising complements of the disclosed natural vertebrate UNC-5 coding sequences. Antisense modulation of the expression of a given vertebrate UNC-5 protein may employ antisense nucleic acids operably linked to gene regulatory sequences. Cells are transfected with a 20 vector comprising a vertebrate UNC-5 sequence with a promoter sequence oriented such that transcription of the gene yields an antisense transcript capable of binding to endogenous vertebrate UNC-5 encoding mRNA. Transcription of the antisense nucleic acid may be constitutive or inducible and the vector may provide for stable extrachromosomal maintenance or integration. Alternatively, single-stranded antisense nucleic acids that bind to 25 genomic DNA or mRNA encoding a given vertebrate UNC-5 protein may be administered to the target cell, in or temporarily isolated from a host, at a concentration that results in a substantial reduction in expression of the targeted protein. An enhancement in vertebrate UNC-5 expression is effected by introducing into the targeted cell type vertebrate UNC-5 30 nucleic acids which increase the functional expression of the corresponding gene products. Such nucleic acids may be vertebrate UNC-5 expression vectors, vectors which upregulate

the functional expression of an endogenous allele, or replacement vectors for targeted correction of mutant alleles. Techniques for introducing the nucleic acids into viable cells are known in the art and include retroviral-based transfection, viral coat protein-liposome mediated transfection, etc.

The invention provides efficient methods of identifying agents, compounds or lead compounds for agents active at the level of a vertebrate UNC-5 modulatable cellular function. 5 Generally, these screening methods involve assaying for compounds which modulate vertebrate UNC-5 interaction with a natural vertebrate UNC-5 binding target. A wide variety of assays for binding agents are provided including labeled *in vitro* protein-protein binding assays, immunoassays, cell based assays, animal based assay, etc. Preferred methods are 10 amenable to automated, cost-effective high throughput screening of chemical libraries for lead compounds. Such libraries encompass candidate agents of numerous chemical classes, though typically they are organic compounds; preferably small organic compounds and are obtained from a wide variety of sources including libraries of synthetic or natural compounds. Identified agents find use in the pharmaceutical industries for animal and human trials; for 15 example, the agents may be derivatized and rescreened in *in vitro* and *in vivo* assays to optimize activity and minimize toxicity for pharmaceutical development.

In vitro binding assays employ a mixture of components including vertebrate UNC-5 protein, which may be part of a fusion product with another peptide or polypeptide, e.g. a tag for detection or anchoring, etc. The assay mixtures comprise a natural extracellular vertebrate 20 UNC-5 binding target, such as a netrin. While native binding targets may be used, it is frequently preferred to use portions (e.g. peptides) thereof so long as the portion provides binding affinity and avidity to the subject vertebrate UNC-5 protein conveniently measurable in the assay. The assay mixture also comprises a candidate pharmacological agent and typically, a variety of other reagents such as salts, buffers, neutral proteins, e.g. albumin, 25 detergents, protease inhibitors, nuclease inhibitors, antimicrobial agents, etc. The mixture components can be added in any order that provides for the requisite bindings and incubations may be performed at any temperature which facilitates optimal binding. The mixture is then incubated under conditions whereby, but for the presence of the candidate pharmacological agent, the vertebrate UNC-5 protein specifically binds the cellular binding target, portion or 30 analog with a reference binding affinity. Incubation periods are likewise selected for optimal binding but also minimized to facilitate rapid, high-throughput screening.

After incubation, the agent-biased binding between the vertebrate UNC-5 protein and one or more binding targets is detected. A separation step is often initially used to separate bound from unbound components. Separation may be effected by precipitation (e.g. TCA precipitation, immunoprecipitation, etc.), immobilization (e.g. on a solid substrate), etc., followed by washing by, for example, membrane filtration, gel chromatography (e.g. gel filtration, affinity, etc.). One of the components usually comprises or is coupled to a label. The label may provide for direct detection such as radioactivity, luminescence, optical or electron density, etc. or indirect detection such as an epitope tag, an enzyme, etc. A variety of methods may be used to detect the label depending on the nature of the label and other assay components, e.g. through optical or electron density, radiative emissions, nonradiative energy transfers, etc. or indirectly detected with antibody conjugates, etc. A difference in the binding affinity of the vertebrate UNC-5 protein to the target in the absence of the agent as compared with the binding affinity in the presence of the agent indicates that the agent modulates the binding of the vertebrate UNC-5 protein to the vertebrate UNC-5 binding target. Analogously, in the cell-based transcription assay also described below, a difference in the vertebrate UNC-5 transcriptional induction in the presence and absence of an agent indicates the agent modulates vertebrate UNC-5-induced transcription. A difference, as used herein, is statistically significant and preferably represents at least a 50%, more preferably at least a 90% difference.

The following experimental section and examples are offered by way of illustration and not by way of limitation.

EXPERIMENTAL

cDNAs encoding two rat homologues of UNC-5, termed UNC5H-1 (SEQ ID NO:1) and UNC5H-2 (SEQ ID NO:2), were isolated from an E18 rat brain cDNA library (see Methods). The predicted proteins (SEQ ID NOS: 3 and 4) show sequence similarity with UNC-5 over their entire lengths, but are more similar to one another (52% identity) than to UNC-5 (28% identity in each case). Like UNC-5¹⁴, both possess two predicted Ig-like domains and two predicted thrombospondin type-1 repeats in their extracellular domains, a predicted membrane spanning region, and a large intracellular domain. The UNC5H proteins also each possess a signal sequence which, curiously, is lacking in UNC-5¹⁴. The predicted topology of the UNC5H proteins in cell membranes was verified using recombinant versions of the proteins expressed

in transfected cells and antibodies directed against the extracellular and intracellular domains (see Methods). The cytoplasmic domains of the two UNC5H proteins do not contain obvious signaling motifs, but do possess a small region of homology to Zona Occludens-1 (ZO-1), a protein that localizes to adherens junctions and is implicated in junction formation^{18, 19}. ZO-1 contains PDZ-domains^{18, 19}, structures implicated in protein clustering²⁰, but the region of homology with UNC-5 homologues corresponds to a unique sequence at the carboxy terminus of ZO-1. The homology between ZO-1 and *C. elegans* UNC-5 is less pronounced (and is not detected by computer BLAST search), but is nonetheless apparent when all four sequences are aligned.

To determine whether the UNC-5 homologues are candidates for receptors involved in neuronal migration or axon guidance, we first examined the sites of expression of *Unc5h-1* and *Unc5h-2* by RNA in situ hybridization in rat embryos. *Unc5h-1* transcripts are detected at early stages of neural tube development in the ventral spinal cord. At embryonic day 11 (E11), when motoneurons are beginning to differentiate in that region²¹, transcripts are present throughout the ventral spinal cord, excluding the midline floor plate region, but are most intense in the ventricular zone and at the lateral edges. At E12, prominent expression is observed in the motor columns, but also extends more dorsally, and is now becoming excluded from the ventricular zone. This more dorsal expression appears transient, as expression by E13 is confined to postmitotic cells in the ventral spinal cord, apparently including the motoneurons. *Unc5h-2* transcripts are not detected at significant levels in the spinal cord until E14, when they are found in the roof plate region. *Unc5h-2* transcripts are, however, detected in developing sensory ganglia that flank the spinal cord, at low levels at E12, and at higher levels by E14. The expression of these two genes is thus observed in regions where differentiating neurons are undergoing axonogenesis, consistent with a possible role in this process.

Expression of these genes is also observed at higher axial levels of the nervous system, as well as in non-neural structures. At E13, *Unc5h-1* is expressed in the basal plate (ventral neural tube) in the hindbrain and midbrain, in the developing hypothalamus and thalamus, and in the pallidum. *Unc5h-2* expression at this stage is detected in the dorsal aspect of the developing optic cup, the nasal pits, apical ridge of the limb bud, urogenital tubercle, and in restricted regions of the midbrain and caudal diencephalon. By E16, *Unc5h-1* mRNA is also detected at high levels in the entorhinal cortex and at lower levels throughout the cortex. *Unc5h-2* is also detected at this stage at low levels in the cortex, and at high levels in hypertrophic

chondrocytes. Expression of the two homologues persists postnatally, with, at postnatal day 10 (P10), continued expression of both at low levels throughout the cortex, expression of both in distinct patterns in the septal area, and high level expression of *Unc5h-1* in the developing hippocampus and entorhinal cortex. In addition, a prominent site of postnatal expression of both genes is in the cerebellum. Both are expressed in the inner granule cell layer, and *Unc5h-2* is in addition expressed in the inner aspect of the external germinal layer, where granule cell precursors differentiate prior to migrating to their final destination in the inner granule cell layer^{22, 23}. Thus, expression of *Unc5h-2* in this region is associated with a prominent cell migration event in the developing cerebellum.

Although the expression patterns of the two UNC5H proteins were suggestive of potential roles in cell or axon migration, to obtain more direct evidence implicating them in mediating responses to netrins we tested whether netrin-1 can bind cells expressing these proteins. Transfected monkey kidney COS-1 cells or human embryonic kidney 293 cells expressing either UNC5H-1 or UNC5H-2 showed significant binding of netrin-1 protein above background, as is also observed for transfected cells expressing the netrin receptors DCC and neogenin, but not for transfected cells expressing TAG-1 or L1, two other members of the Ig superfamily¹³. In these experiments, binding was performed in the presence of soluble heparin, which eliminates non-specific binding of netrin-1 to the cells¹³ but does not evidently prevent binding to the UNC5 homologues. To verify, in the case of UNC5H-2, that exogenously added heparin is not required for the interaction, we generated a soluble protein comprising the extracellular domain of UNC5H-2 fused to the constant region (Fc) of a human immunoglobulin molecule. This UNC5H-2-Fc fusion protein bound transfected 293 cells expressing netrin-1 (some of which remains associated with the surface of these cells^{3, 10}) in the absence of added heparin but did not show binding to non-transfected cells, nor to cells expressing UNC5H-2 itself, DCC, or neogenin. The UNC5H-2-Fc fusion also did not bind transfected cells expressing F-spondin, an adhesive extracellular matrix protein made by floor plate cells²⁴, or Semaphorin III, a chemorepellent for sensory axons at the stages that *Unc5h-2* is expressed in sensory ganglia²⁵. Both of these proteins, like netrin-1, are secreted but partition between cell surfaces and the soluble fraction^{24, 26}. Thus, the interaction between netrin-1 and UNC5H-2 appears specific, and does not require heparin nor reflect a generalized interaction with proteins that associate non-specifically with cell surfaces.

The affinity of UNC-5 homologues for netrin-1 was estimated in equilibrium binding

experiments using netrin(VIoV)-Fc, a fusion of the amino terminal two-thirds of netrin-1 to the constant portion of human IgG¹³. This netrin-1 derivative is bioactive but, unlike netrin-1, does not aggregate at high concentrations, and it binds DCC with a Kd comparable to that of full length netrin-1¹³. Specific binding of netrin (VIoV)-Fc to each of the three UNC5 homologues showed saturation and the binding curves were fitted to the Hill equation, yielding Kd values of 5 19 ± 0.8 nM and 3.4 ± 1.0 nM for UNC5H1 and UNC5H2 respectively. These values are comparable to the Kd for the DCC-netrin (VIoV-Fc) interaction (~5 nM), and are consistent with the effective dose for the axon outgrowth promoting effects of netrin-1^{2, 13}.

Establishing the involvement of these vertebrate UNC5H proteins in cell migration and axon guidance will require perturbing their functions *in vivo*. In the meantime, however, our 10 results are at least consistent with such an involvement, as these homologues are expressed by some populations of cells that are undergoing migrations or extending axons. For example, *Unc5h1* is expressed by spinal motoneurons, whose axons are repelled *in vitro* by floor plate cells²⁷, and whose outgrowth *in vitro* can be suppressed by netrin-1. It is also expressed in the 15 region of trochlear motoneurons, which can be repelled by netrin-1⁴. Both *Unc5h* genes are also expressed in the developing cerebellum, which is a site of extensive cell migration.

Although the *in vivo* functions of the UNC-5 homologues described here remain to be determined, our evidence that vertebrate UNC5H proteins bind netrin-1 provides direct support for the idea that members of this new subfamily of the Ig superfamily are netrin receptors. This idea was first proposed for *C. elegans* UNC-5, based on the findings that *unc-5* is required cell-autonomously for dorsal migrations that require the function of the netrin UNC-6¹⁴, and that 20 ectopic expression of *unc-5* in neurons that normally project longitudinally or ventrally can steer their axons dorsally¹⁷. Although consistent with the possibility that UNC-5 is an UNC-6 receptor, these results are also consistent with a role for UNC-5 in modifying the function of a distinct UNC-6 receptor. The possibility of a modifier function was made more plausible by 25 evidence that the DCC homologue UNC-40, which is a putative UNC-6 receptor involved in ventral migrations¹¹, is expressed by axons that project dorsally and is required for those projections^{11, 15, 16}, suggesting that UNC-5 might function by switching an attractive netrin receptor (UNC-40) into a repulsive netrin receptor. However, our results suggest that UNC-5 also functions directly as a netrin receptor. A model in which UNC-40 and UNC-5 can form a 30 receptor complex but UNC-5 can also function alone in transducing the UNC-6 netrin signal provides an explanation for the observation that loss of *unc-40* function results in a much less

severe phenotype for dorsal migrations than do either loss of *unc-5* or loss of *unc-6* function¹⁵.
16.

Recent studies have demonstrated a remarkable phylogenetic conservation in function of netrin proteins in guiding axons towards a source of netrin at the midline of the nervous systems of nematodes, flies and vertebrates^{1, 7, 8, 9}, as well as a conserved role for members of the DCC 5 subfamily of the Ig superfamily in mediating the axonal responses that underlie those guidance events^{11, 12, 13}. The identification of vertebrate homologues of UNC-5, and the evidence that they are netrin-binding proteins, suggests that the signaling mechanisms through which netrins elicit repulsive responses are also conserved.

10 *Isolation of rat UNC-5 homologues, and in situ hybridization.* A search of the human expressed sequence tag (EST) databases revealed a small sequence (Genbank accession number R11880) with distant similarity to the carboxy-terminal portion of UNC-5. The corresponding cDNA fragment, amplified by polymerase chain reaction from an embryonic human brain cDNA library (Stratagene), was used to screen the library, resulting in the isolation of a 3.8 kB cDNA clone comprising all but the first 440 nt of the coding region of the human homologue of UNC5H1. Non-overlapping probes from this cDNA were used to screen an E18 rat brain library 15 (gift of S. Nakanishi), leading to isolation of seven partial and one full length UNC5H1 cDNA and one full length UNC5H2 cDNA. Additional screens of E13 rat dorsal and ventral spinal cord libraries resulted in isolation of a second full length UNC5H2 cDNA as well as a nearly full length UNC5H1 cDNA. Sequencing was performed on a Licor (L4000) automated sequencer 20 as well as by ³³P cycle sequencing. Genbank accession numbers are U87305 and U87306 for rUNC5H1 and rUNC5H2 respectively. RNA *in situ* hybridization was performed as described¹³.

25 *Antibodies, expression constructs and immunohistochemistry.* Rabbit polyclonal antisera were raised to a peptide corresponding to a sequence (YLRKNFEQEPLAKE, SEQ ID NO:7, residues 148-161) in the extracellular domain of UNC5H-2 that is almost completely conserved in UNC5H-1 (one amino acid substitution), and to peptides corresponding to unique sequences in the cytoplasmic domains of UNC5H-1 (GEPSPDSWSLRLKKQ, SEQ ID NO:5, residues 580-594) and UNC5H-2 (EARQQDDGDLNSLASA, SEQ ID NO:7, residues 909-924). Antisera 30 were affinity-purified on the respective peptides (Quality Controlled Biochemicals). cDNAs for the various constructs were subcloned into the COS cell expression vector pMT21 and the 293-EBNA cell expression vector pCEP4 (Invitrogen), and transiently transfected into those cells using lipofectamine. The antiserum to the extracellular peptide can detect both UNC5H proteins

expressed in transfected cells without cell permeabilization, whereas the antisera directed against the cytoplasmic domain peptides detected their respective proteins after cell permeabilization. Netrin-1 protein was produced, purified, used and visualized in binding assays as described¹³, except that a monoclonal antibody (9E10)²⁹ directed to a C-terminal myc-epitope tag was used to detect recombinant netrin-1, and heparin was used at 1 μ g/ml. A 293-EBNA cell line stably expressing the UNC5H-2-Fc fusion was derived and maintained as described^{10, 13}. The fusion protein was purified from serum-free medium conditioned for seven days by affinity chromatography on protein A agarose. The 293 cell line expressing netrin-1 was as described¹³. Binding of the UNC5H-2-Fc fusion to this line was visualized using a Cy3-conjugated secondary antibody (Jackson Immunoresearch) directed against human Fc.

5 10 *References*

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3. Kennedy, T. E., Serafini, T., de la Torre, J. R. & Tessier-Lavigne, M. *Cell* 78, 425-35 (1994).
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17. Hamelin, M., Zhou, Y., Su, M. W., Scott, I. M. & Culotti, J. G. *Nature* 364, 327-30 (1993).
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EXAMPLES

1. Protocol for high throughput vertebrate UNC-5 - netrin binding assay.
- 10 A. Reagents:
 - Neutralite Avidin: 20 µg/ml in PBS.
 - Blocking buffer: 5% BSA, 0.5% Tween 20 in PBS; 1 hour at room temperature.
 - Assay Buffer: 100 mM KCl, 20 mM HEPES pH 7.6, 1 mM MgCl₂, 1% glycerol, 0.5% NP-40, 50 mM b-mercaptoethanol, 1 mg/ml BSA, cocktail of protease inhibitors.
 - 15 - ³³P vertebrate UNC-5 protein 10x stock: 10⁻⁸ - 10⁻⁶ M "cold" vertebrate UNC-5 supplemented with 200,000-250,000 cpm of labeled vertebrate UNC-51 (Beckman counter). Place in the 4°C microfridge during screening.
 - Protease inhibitor cocktail (1000X): 10 mg Trypsin Inhibitor (BMB # 109894), 10 mg Aprotinin (BMB # 236624), 25 mg Benzamidine (Sigma # B-6506), 25 mg Leupeptin (BMB # 20 1017128), 10 mg APMSF (BMB # 917575), and 2mM NaVo₃ (Sigma # S-6508) in 10 ml of PBS.
 - netrin-1: 10⁻⁷ - 10⁻⁵ M biotinylated netrin-1 in PBS.
- 20 B. Preparation of assay plates:
 - Coat with 120 µl of stock N-Avidin per well overnight at 4°C.
 - Wash 2 times with 200 µl PBS.
 - 25 - Block with 150 µl of blocking buffer.
 - Wash 2 times with 200 µl PBS.
- C. Assay:
 - Add 40 µl assay buffer/well.
 - Add 10 µl compound or extract.
 - 30 - Add 10 µl ³³P-UNC-5 (20-25,000 cpm/0.1-10 pmoles/well = 10⁻⁹- 10⁻⁷ M final conc).
 - Shake at 25°C for 15 minutes.

- Incubate additional 45 minutes at 25°C.
- Add 40 μ M biotinylated netrin-1 (0.1-10 pmoles/40 μ l in assay buffer)
- Incubate 1 hour at room temperature.
- Stop the reaction by washing 4 times with 200 μ M PBS.
- Add 150 μ M scintillation cocktail.

5

- Count in Topcount.

D. Controls for all assays (located on each plate):

- a. Non-specific binding
- b. Soluble (non-biotinylated netrin-1) at 80% inhibition.

10 All publications and patent applications cited in this specification are herein incorporated by reference as if each individual publication or patent application were specifically and individually indicated to be incorporated by reference. Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be readily apparent to those of ordinary skill in the art in light of the 15 teachings of this invention that certain changes and modifications may be made thereto without departing from the spirit or scope of the appended claims.

SEQUENCE LISTING

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5

(ii) TITLE OF INVENTION: Netrin Receptors

(iii) NUMBER OF SEQUENCES: 8

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10

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15

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(v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk

(B) COMPUTER: IBM PC compatible

(C) OPERATING SYSTEM: PC-DOS/MS-DOS

20

(D) SOFTWARE: PatentIn Release #1.0, Version #1.30

(vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER: US

(B) FILING DATE:

(C) CLASSIFICATION:

25

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30

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(A) TELEPHONE: (415) 343-4341

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(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

35

(A) LENGTH: 3014 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: double

(D) TOPOLOGY: linear

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(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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CGTGGTTCGG	GTGCCAGCA	GAGTGCCACG	GTGGCCAATC	CAGTGCCCGG	TGCCAACCCC	120
GACCTGCTGC	CCCACTTCCCT	GGTAGAGCCT	GAGGACGTGT	ACATTGTCAA	GAACAAGCCG	180
GTGTTGTTGG	TGTGCAAGGC	TGTGCCTGCC	ACCCAGATCT	TCTTCAAGTG	CAATGGGAA	240

	TGGGTCCGCC AGGTCGATCA CGTAATTGAA CGCAGCACCG ACAGCAGCAG CGGATTGCCA	300
	ACCATGGAGG TCCGTATCAA CGTATCGAGG CAGCAGGTAG AGAAAGTGTG TGGGCTGGAG	360
	GAATACTGGT GCCAGTGTGT GGCATGGAGC TCCTCGGGTA CCACCAAAAG TCAGAAGGCC	420
	TACATCCGGA TTGCTTATTT GCGCAAGAAC TTTGAGCAGG AGCCACTGGC CAAGGAAGTG	480
5	TCACTGGAGC AAGGCATTGT ACTACCTTGT CGCCCCCAG AAGGAATCCC CCCAGCTGAG	540
	GTGGAGTGGC TTGAAATGA GGACCTCGT GACCCCTCCC TCGATCCAA TGTGTACATC	600
	ACGCGGGAGC ACAGCCTAGT CGTGCCTAG GCCCCCTGG CCGACACGGC CAACTACACC	660
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	GTGAACGGTG GGTGGTCGAC GTGGACTGAG TGGTCCGTCT GCAGCGCCAG CTGTGGCGT	780
	GGCTGGCAGA AACGGAGCCG GAGCTGCACC AACCCGGCAC CTCTCAACGG GGGCGCCTTC	840
10	TGTGAGGGC AGAATGTCCA GAAAACAGCC TGCCTCACTC TGTGCCAGT GGATGGGAGC	900
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15	CTCATTACT GTCGCAAGAA GGAAGGGCTG GACTCCGATG TGGCCGACTC GTCCATCCTC	1200
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	CAGGATGGAC CCAGCCCCAA GTTCCAGCTC TCTAATGGTC ACCTGCTCAG CCCACTGGGG	1380
	AGTGGCCGCC ATACGTTGCA CCACAGCTCA CCCACCTCTG AGGCTGAGGA CTTCGTCTCC	1440
20	CGCCTCTCCA CCCAAACTA CTTTCGTTCC CTGCCCCGCG GCACCAGCAA CATGGCCTAC	1500
	GGGACCTTCA ACTTCCTCGG GGGCCGGCTG ATGATCCCTA ATACGGGGAT CAGCCTCCTC	1560
	ATACCCCCGG ATGCCATCCC CCGAGGAAAG ATCTACGAGA TCTACCTCAC ACTGCACAAG	1620
	CCAGAAAGACG TGAGGTTGCC CCTAGCTGGC TGTCAAGACCC TGCTGAGTCC AGTCGTTAGC	1680
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	CTCAGCGTGG CTGCCACCAA GGCCTCAGG CTCCCTCTGT TTGCTCCCGT GGCCTGTACG	1980
	TCCCTTGAGT ACAACATCCG AGTGTACTGC CTACACGACA CCCACGACGC TCTCAAGGAG	2040
30	GTGGTGCAGC TGGAGAAGCA GCTAGGTGGA CAGCTGATCC AGGAGCCTCG CGTCCTGCAC	2100
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	GCCAGTCTGG ACCCACCCCTG CAGCCGGGGC GCCGACTGGA GAACTCTAGC CCAGAAACTT	2520
	CACCTGGACA GCCATCTTAG CTTCTTGTCC TCCAAGCCCA GCCCTACAGC CATGATCCTC	2580
	AACCTATGGG AGGCACGGCA CTTCCCCAAC GGCAACCTCG GCCAGCTGGC AGCAGCTGTG	2640
40	GCCGGACTGG GCCAACCGAGA TGCTGGCCTC TTCACGGTGT CGGAGGCCGA GTGTTGAGAC	2700
	CAGCCAGGCC GGTAATGCCT ACATTCTCAC CAGCTTGAC ACCTGCCAGG GACAGGCCAA	2760
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	TGAAGTTGTC CCTCGATGCT GGTCTTCAG ACCCTGCCA AACTCCATCC CTCCATGGCC	2880
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(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

5 (A) LENGTH: 1787 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: double
 (D) TOPOLOGY: linear

10 (ii) MOLECULE TYPE: cDNA
 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

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AGGCTTCAG CCCGTCAGCA TCTAAGCCCA GCAAAGCAGA CAACCCCCAT CTGCTCACCA	240
15 TCCAGCCCGA CCTCAGCACC ACCACCACCA CCTACCAGGG CAGTCTCTGT CCCCAGCAGG	300
ATGGGCCCAG CCCCCAAGTTC CAGCTCACCA ATGGGCACCT GCTCAGCCCC CTGGGTGGCG	360
GCCGCCACAC ACTGCACCAC AGCTCTCCCA CCTCTGAGGC CGAGGAGTTC GTCTCCCGCC	420
TCTCCACCCA GAACTACTTC CGCTCCCTGC CCCGAGGCAC CAGCAACATG ACCTATGGGA	480
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30 CTCCTTGTCA GCTACCAAGGA GATCCCCTTT TATCACATCT GGAATGGCAC GCAGCGGTAC	1200
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(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 2831 base pairs

(B) TYPE: nucleic acid
 (C) STRANDEDNESS: double
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

5	ATGAGGGCCC GGAGCGGCGG GGCGCCTGCT GTGGCGCTGC TGCTCTGCTG GGATCCGACA	60
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	CCCGCGGAGC AGCTGCCTCA CTTCCCTGCTG GAACCAAGAGG ATGCCTACAT CGTAAAGAAC	180
	AAGCCAGTGG AATTGCACTG CCGAGCCTTC CCTGCCACAC AGATCTACTT CAAGTGTAAAT	240
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10	TTGCGAATAAC GAGAGGTGCA GATAGAGGTG TCGCGGCAGC AGGTGGAGGA ACTTTTTGGG	360
	CTCGAGGACT ACTGGGTGTCA GTGCGTGGCC TGGAGCTCTT CGGGAACACC CAAGAGTCGC	420
	CGAGCCTACA TCCGCATTGC CTACTTGCGC AAGAACTTTG ACCAGGAGCC TCTGGCGAAG	480
	GAGGTACCCCT TGGATCATGA GGTCCCTCTG CAGTGGCGCC CACCAAGAGGG AGTGCCTGTG	540
	GCTGAGGTGG AATGGCTCAA GAATGAAGAT GTCATCGATC CCGCTCAGGA CACTAACTTC	600
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	GGCCGAGGTT GGCAGAACG TACTAGGACC TGCACCAACC CAGCCCCACT CAATGGAGGT	840
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20	GGAGCGTGGGA CTGAGTGGAG CAAGTGGTCC GCCTGCAGCA CAGAGTGTGC GCACTGGCGC	960
	AGCCGCGAGT GCATGGCACC GCCGCCCCAG AACGGAGGCC GTGACTGCAG CGGGACGCTA	1020
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25	TGCCGGGACT TCGACACGGA CATCACTGAC TCCTCTGCTG CCCTCACTGG TGGTTTCCAC	1260
	CCCGTCAACT TCAAGACTGC AAGGCCACAGC AACCCACAGC TCCTGCACCC ATCCGCCCCCT	1320
	CCGGACCTAA CGGCCAGTGC TGGCATCTAC CGCGGACCTG TGTATGCCCT GCAGGACTCT	1380
	GCCGACAAGA TCCCTATGAC TAATTCAACCC CTTCTGGATC CCTTGCCCAG CCTCAAGATC	1440
	AAGGTCTATG ACTCCAGCAC CATCGGCTCT GGGGCTGGCC TGGCTGATGG AGCCGACCTG	1500
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	CGTATCAACA AGACTGAAAG CACCCCTCCA CTTTCGGAAG GTTCCCAGAC AGTATTGAGC	1800
35	CCCTCGGTGA CCTGCGGGCC CACGGGCCTC CTCCCTGTGCC GCCCTGTGTG CTCCTACTGTG	1860
	CCCCACTGTG CTGAAGTCAT TGCCGGAGAC TGGATCTTCC AGCTCAAGAC CCAGGGCCCAT	1920
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	TGCCAGCTAG AGGCTAAATC CTGCCACATC CTGTTGGACC AGCTGGGTAC CTACGTGTTC	2040
	ACGGGCGAGT CCTACTCCCG CTCCGCAGTC AAGCGGCTCC AGCTAGCCAT CTTCGCCCCA	2100
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	GCACTGAAGG AGGTCCCTAGA GCTGGAGAGG ACTCTGGGTG GCTACTTTGGT GGAGGAGCCC	2220
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CCACTGAGTT CACCTGTAAG GTCTGCGTGC GGCAGGTAGA AGGGGAAGGC CAGATTTCC	2460
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CTGGCAATGC TGCCACCAACA CAGCTGGAC CCTATGCCCT CAAGATACCA CTGTCCATCC	2580
GCCAGAAAGAT CTGCAACAGC CTGGACGCC CCAACTCACG GGGCAATGAC TGGCGGCTGT	2640
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5 CAGGCGTGTAT CTTAGACCTC TGGGAAGCTC GGCAGCAGGA TGATGGGGAC CTCAACAGCC	2760
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GCGATTGCTG A	2831

(2) INFORMATION FOR SEQ ID NO:4:

10 (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 305 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

15 (ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

TGGATGAGGA GACCCTGAAC ACACCCTGCT ACTGCAGCTG GAGCCCAGGG CCTGTACATC	60
CTGCTGGACC AGCTGGGCAC CTACGTTTTC ACGGGGAGT CCTATTCCCG CTCAGCAGTC	120
AAGCGGCTCC AGCTGGCCGT TTGCCCCCG CCCTCTGCAC CTCCCTGGAG TACAGCCTCC	180
20 GGGTCTACTG CCTGGAGGAC ACGCCTGTAG CACTGAAGGA GGTGCTGGAG CTGGAGCGGA	240
CTCTGGCGG ATACTTGGTG GAGGAGCCGA AACCGCTAAT GTTCAAGGAC AGTTACCACA	300
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(2) INFORMATION FOR SEQ ID NO:5:

25 (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 898 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: not relevant
- (D) TOPOLOGY: not relevant

30 (ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

Met Ala Val Arg Pro Gly Leu Trp Pro Val Leu Leu Gly Ile Val Leu	
1 5 10 15	

Ala Ala Trp Leu Arg Gly Ser Gly Ala Gln Gln Ser Ala Thr Val Ala	
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35 Asn Pro Val Pro Gly Ala Asn Pro Asp Leu Leu Pro His Phe Leu Val	
35 40 45	

50 Glu Pro Glu Asp Val Tyr Ile Val Lys Asn Lys Pro Val Leu Leu Val	
50 55 60	

40 65 Cys Lys Ala Val Pro Ala Thr Gln Ile Phe Phe Lys Cys Asn Gly Glu	
65 70 75 80	

Trp Val Arg Gln Val Asp His Val Ile Glu Arg Ser Thr Asp Ser Ser	
85 90 95	

Ser Gly Leu Pro Thr Met Glu Val Arg Ile Asn Val Ser Arg Gln Gln	
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	100	105	110
	Val Glu Lys Val Phe Gly Leu Glu Glu Tyr Trp Cys Gln Cys Val Ala		
	115	120	125
	Trp Ser Ser Ser Gly Thr Thr Lys Ser Gln Lys Ala Tyr Ile Arg Ile		
5	130	135	140
	Ala Tyr Leu Arg Lys Asn Phe Glu Gln Glu Pro Leu Ala Lys Glu Val		
	145	150	155
	Ser Leu Glu Gln Gly Ile Val Leu Pro Cys Arg Pro Pro Glu Gly Ile		
	165	170	175
10	Pro Pro Ala Glu Val Glu Trp Leu Arg Asn Glu Asp Leu Val Asp Pro		
	180	185	190
	Ser Leu Asp Pro Asn Val Tyr Ile Thr Arg Glu His Ser Leu Val Val		
	195	200	205
	Arg Gln Ala Arg Leu Ala Asp Thr Ala Asn Tyr Thr Cys Val Ala Lys		
	210	215	220
15	Asn Ile Val Ala Arg Arg Ser Thr Ser Ala Ala Val Ile Val Tyr		
	225	230	235
	Val Asn Gly Gly Trp Ser Thr Trp Thr Glu Trp Ser Val Cys Ser Ala		
	245	250	255
20	Ser Cys Gly Arg Gly Trp Gln Lys Arg Ser Arg Ser Cys Thr Asn Pro		
	260	265	270
	Ala Pro Leu Asn Gly Gly Ala Phe Cys Glu Gly Gln Asn Val Gln Lys		
	275	280	285
	Thr Ala Cys Ala Thr Leu Cys Pro Val Asp Gly Ser Trp Ser Ser Trp		
	290	295	300
25	Ser Lys Trp Ser Ala Cys Gly Leu Asp Cys Thr His Trp Arg Ser Arg		
	305	310	315
	Glu Cys Ser Asp Pro Ala Pro Arg Asn Gly Gly Glu Glu Cys Arg Gly		
	325	330	335
30	Ala Asp Leu Asp Thr Arg Asn Cys Thr Ser Asp Leu Cys Leu His Thr		
	340	345	350
	Ala Ser Cys Pro Glu Asp Val Ala Leu Tyr Ile Gly Leu Val Ala Val		
	355	360	365
35	Ala Val Cys Leu Phe Leu Leu Leu Ala Leu Gly Leu Ile Tyr Cys		
	370	375	380
	Arg Lys Lys Glu Gly Leu Asp Ser Asp Val Ala Asp Ser Ser Ile Leu		
	385	390	395
	Thr Ser Gly Phe Gln Pro Val Ser Ile Lys Pro Ser Lys Ala Asp Asn		
	405	410	415
40	Pro His Leu Leu Thr Ile Gln Pro Asp Leu Ser Thr Thr Thr Thr		
	420	425	430
	Tyr Gln Gly Ser Leu Cys Ser Arg Gln Asp Gly Pro Ser Pro Lys Phe		
	435	440	445
	Gln Leu Ser Asn Gly His Leu Leu Ser Pro Leu Gly Ser Gly Arg His		

	450	455	460
	Thr Leu His His Ser Ser Pro Thr Ser Glu Ala Glu Asp Phe Val Ser		
	465	470	475
	Arg Leu Ser Thr Gln Asn Tyr Phe Arg Ser Leu Pro Arg Gly Thr Ser		480
	485	490	495
5	Asn Met Ala Tyr Gly Thr Phe Asn Phe Leu Gly Gly Arg Leu Met Ile		
	500	505	510
	Pro Asn Thr Gly Ile Ser Leu Leu Ile Pro Pro Asp Ala Ile Pro Arg		
	515	520	525
	Gly Lys Ile Tyr Glu Ile Tyr Leu Thr Leu His Lys Pro Glu Asp Val		
10	530	535	540
	Arg Leu Pro Leu Ala Gly Cys Gln Thr Leu Leu Ser Pro Val Val Ser		
	545	550	555
	Cys Gly Pro Pro Gly Val Leu Leu Thr Arg Pro Val Ile Leu Ala Met		560
	565	570	575
15	Asp His Cys Gly Glu Pro Ser Pro Asp Ser Trp Ser Leu Arg Leu Lys		
	580	585	590
	Lys Gln Ser Cys Glu Gly Ser Trp Glu Asp Val Leu His Leu Gly Glu		
	595	600	605
	Glu Ser Pro Ser His Leu Tyr Tyr Cys Gln Leu Glu Ala Gly Ala Cys		
20	610	615	620
	Tyr Val Phe Thr Glu Gln Leu Gly Arg Phe Ala Leu Val Gly Glu Ala		
	625	630	635
	Leu Ser Val Ala Ala Thr Lys Arg Leu Arg Leu Leu Phe Ala Pro		
	645	650	655
25	Val Ala Cys Thr Ser Leu Glu Tyr Asn Ile Arg Val Tyr Cys Leu His		
	660	665	670
	Asp Thr His Asp Ala Leu Lys Glu Val Val Gln Leu Glu Lys Gln Leu		
	675	680	685
	Gly Gly Gln Leu Ile Gln Glu Pro Arg Val Leu His Phe Lys Asp Ser		
30	690	695	700
	Tyr His Asn Leu Arg Leu Ser Ile His Asp Val Pro Ser Ser Leu Trp		
	705	710	715
	Lys Ser Lys Leu Leu Val Ser Tyr Gln Glu Ile Pro Phe Tyr His Ile		720
	725	730	735
35	Trp Asn Gly Thr Gln Gln Tyr Leu His Cys Thr Phe Thr Leu Glu Arg		
	740	745	750
	Ile Asn Ala Ser Thr Ser Asp Leu Ala Cys Lys Val Trp Val Trp Gln		
	755	760	765
	Val Glu Gly Asp Gly Gln Ser Phe Asn Ile Asn Phe Asn Ile Thr Lys		
40	770	775	780
	Asp Thr Arg Phe Ala Glu Leu Leu Ala Leu Glu Ser Glu Gly Gly Val		
	785	790	795
	Pro Ala Leu Val Gly Pro Ser Ala Phe Lys Ile Pro Phe Leu Ile Arg		800
	805	810	815

Gln Lys Ile Ile Ala Ser Leu Asp Pro Pro Cys Ser Arg Gly Ala Asp
 820 825 830
 Trp Arg Thr Leu Ala Gln Lys Leu His Leu Asp Ser His Leu Ser Phe
 835 840 845
 Phe Ala Ser Lys Pro Ser Pro Thr Ala Met Ile Leu Asn Leu Trp Glu
 5 850 855 860
 Ala Arg His Phe Pro Asn Gly Asn Leu Gly Gln Leu Ala Ala Val
 865 870 875 880
 Ala Gly Leu Gly Gln Pro Asp Ala Gly Leu Phe Thr Val Ser Glu Ala
 885 890 895
 10 Glu Cys

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 557 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS: not relevant

(D) TOPOLOGY: not relevant

(iii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

20 Asn Cys Thr Ser Asp Leu Xaa Val His Thr Ala Ser Gly Pro Glu Asp
 1 5 10 15
 Val Ala Leu Tyr Val Gly Leu Ile Ala Val Ala Val Cys Leu Val Leu
 20 25 30
 Leu Leu Leu Val Leu Ile Leu Val Tyr Cys Arg Lys Lys Glu Gly Leu
 25 35 40 45
 Asp Ser Asp Val Ala Asp Ser Ser Ile Leu Thr Ser Gly Phe Gln Pro
 50 55 60
 Val Ser Ile Lys Pro Ser Lys Ala Asp Asn Pro His Leu Leu Thr Ile
 65 70 75 80
 30 Gln Pro Asp Leu Ser Thr Thr Thr Thr Tyr Gln Gly Ser Leu Cys
 85 90 95
 Pro Arg Gln Asp Gly Pro Ser Pro Lys Phe Gln Leu Thr Asn Gly His
 100 105 110
 Leu Leu Ser Pro Leu Gly Gly Arg His Thr Leu His His Ser Ser
 35 115 120 125
 Pro Thr Ser Glu Ala Glu Glu Phe Val Ser Arg Leu Ser Thr Gln Asn
 130 135 140
 Tyr Phe Arg Ser Leu Pro Arg Gly Thr Ser Asn Met Thr Tyr Gly Thr
 145 150 155 160
 40 Phe Asn Phe Leu Gly Gly Arg Leu Met Ile Pro Asn Thr Gly Ile Ser
 165 170 175
 Leu Leu Ile Pro Pro Asp Ala Ile Pro Arg Gly Lys Ile Tyr Glu Ile
 180 185 190
 Tyr Leu Thr Leu His Lys Pro Glu Asp Val Arg Leu Pro Leu Ala Gly

	195	200	205
	Cys Gln Thr Leu Leu Ser Pro Ile Val Ser Cys Gly Pro Pro Gly Val		
	210	215	220
	Leu Leu Thr Arg Pro Val Ile Leu Ala Met Asp His Cys Gly Glu Pro		
5	225	230	235
	Ser Pro Asp Ser Trp Ser Leu Ala Leu Lys Lys Gln Ser Cys Glu Gly		
	245	250	255
	Ser Trp Glu Asp Val Leu His Leu Gly Glu Glu Ala Pro Ser His Leu		
	260	265	270
10	Tyr Tyr Cys Gln Leu Glu Ala Ser Ala Cys Tyr Val Phe Thr Glu Gln		
	275	280	285
	Leu Gly Arg Phe Ala Leu Val Gly Glu Ala Leu Ser Val Ala Ala Ala		
	290	295	300
	Lys Arg Leu Lys Leu Leu Phe Ala Pro Val Ala Cys Thr Ser Leu		
15	305	310	315
	Glu Tyr Asn Ile Arg Val Tyr Cys Leu His Asp Thr His Asp Ala Leu		
	325	330	335
	Lys Glu Val Val Gln Leu Glu Lys Gln Leu Gly Gly Gln Leu Ile Gln		
	340	345	350
20	Glu Pro Arg Val Leu His Leu Xaa Asp Ser Tyr His Asn Leu Xaa Leu		
	355	360	365
	Ser Xaa His Asp Val Pro Ser Ser Leu Trp Lys Ser Lys Leu Leu Val		
	370	375	380
	Ser Tyr Gln Glu Ile Pro Phe Tyr His Ile Trp Asn Gly Thr Gln Arg		
25	385	390	395
	Tyr Leu His Cys Thr Phe Thr Leu Glu Arg Val Ser Pro Ser Thr Ser		
	405	410	415
	Asp Leu Ala Cys Lys Leu Trp Val Trp Gln Val Glu Gly Asp Gly Gln		
	420	425	430
30	Ser Phe Ser Ile Asn Phe Asn Ile Thr Lys Asp Thr Arg Phe Ala Glu		
	435	440	445
	Leu Leu Ala Leu Glu Ser Glu Ala Gly Val Pro Ala Leu Val Gly Pro		
	450	455	460
	Ser Ala Phe Lys Ile Pro Phe Leu Ile Arg Gln Lys Ile Ile Ser Ser		
35	465	470	475
	Leu Asp Pro Pro Cys Arg Arg Gly Ala Asp Trp Arg Thr Leu Ala Gln		
	485	490	495
	Lys Leu His Leu Asp Ser His Leu Ser Phe Phe Ala Ser Lys Pro Ser		
	500	505	510
40	Pro Thr Ala Met Ile Leu Asn Leu Trp Glu Ala Arg His Phe Pro Asn		
	515	520	525
	Gly Asn Leu Ser Gln Leu Ala Ala Val Ala Gly Thr Xaa Pro Ala		
	530	535	540
	Gly Arg Trp Leu Leu Ser Gln Cys Ser Glu Ala Glu Cys		
	545	550	555

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 943 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS: not relevant

(D) TOPOLOGY: not relevant

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

Met Arg Ala Arg Ser Gly Gly Ala Ala Ala Val Ala Leu Leu Leu Cys

1 5 10 15

Trp Asp Pro Thr Pro Ser Leu Ala Gly Ile Asp Ser Gly Ala Gln Gly
20 25 30Leu Pro Asp Ser Phe Pro Ser Ala Pro Ala Glu Gln Leu Pro His Phe
35 40 45Leu Leu Glu Pro Glu Asp Ala Tyr Ile Val Lys Asn Lys Pro Val Glu
50 55 60Leu His Cys Arg Ala Phe Pro Ala Thr Gln Ile Tyr Phe Lys Cys Asn
65 70 75 80Gly Glu Trp Val Ser Gln Lys Gly His Val Thr Gln Glu Ser Leu Asp
85 90 95Glu Ala Thr Gly Leu Arg Ile Arg Glu Val Gln Ile Glu Val Ser Arg
100 105 110Gln Gln Val Glu Glu Leu Phe Gly Leu Glu Asp Tyr Trp Cys Gln Cys
115 120 125Val Ala Trp Ser Ser Ser Gly Thr Thr Lys Ser Arg Arg Ala Tyr Ile
130 135 140Arg Ile Ala Tyr Leu Arg Lys Asn Phe Asp Gln Glu Pro Leu Ala Lys
145 150 155 160Glu Val Pro Leu Asp His Glu Val Leu Leu Gln Cys Arg Pro Pro Glu
165 170 175Gly Val Pro Val Ala Glu Val Glu Trp Leu Lys Asn Glu Asp Val Ile
180 185 190Asp Pro Ala Gln Asp Thr Asn Phe Leu Leu Thr Ile Asp His Asn Leu
195 200 205Ile Ile Arg Gln Ala Arg Leu Ser Asp Thr Ala Asn Tyr Thr Cys Val
210 215 220Ala Lys Asn Ile Val Ala Lys Arg Arg Ser Thr Thr Ala Thr Val Ile
225 230 235 240Val Tyr Val Asn Gly Gly Trp Ser Ser Trp Ala Glu Trp Ser Pro Cys
245 250 255Ser Asn Arg Cys Gly Arg Gly Trp Gln Lys Arg Thr Arg Thr Cys Thr
260 265 270Asn Pro Ala Pro Leu Asn Gly Gly Ala Phe Cys Glu Gly Gln Ala Cys
275 280 285

Gln Lys Thr Ala Cys Thr Thr Val Cys Pro Val Asp Gly Ala Trp Thr
 290 295 300
 Glu Trp Ser Lys Trp Ser Ala Cys Ser Thr Glu Cys Ala His Trp Arg
 305 310 315 320
 5 Ser Arg Glu Cys Met Ala Pro Pro Pro Gln Asn Gly Gly Arg Asp Cys
 325 330 335
 Ser Gly Thr Leu Leu Asp Ser Lys Asn Cys Thr Asp Gly Leu Cys Val
 340 345 350
 Leu Asn Gln Arg Thr Leu Asn Asp Pro Lys Ser Arg Pro Leu Glu Pro
 355 360 365
 10 Ser Gly Asp Val Ala Leu Tyr Ala Gly Leu Val Val Ala Val Phe Val
 370 375 380
 Val Leu Ala Val Leu Met Ala Val Gly Val Ile Val Tyr Arg Arg Asn
 385 390 395 400
 15 Cys Arg Asp Phe Asp Thr Asp Ile Thr Asp Ser Ser Ala Ala Leu Thr
 405 410 415
 Gly Gly Phe His Pro Val Asn Phe Lys Thr Ala Arg Pro Ser Asn Pro
 420 425 430
 Gln Leu Leu His Pro Ser Ala Pro Pro Asp Leu Thr Ala Ser Ala Gly
 435 440 445
 20 Ile Tyr Arg Gly Pro Val Tyr Ala Leu Gln Asp Ser Ala Asp Lys Ile
 450 455 460
 Pro Met Thr Asn Ser Pro Leu Leu Asp Pro Leu Pro Ser Leu Lys Ile
 465 470 475 480
 25 Lys Val Tyr Asp Ser Ser Thr Ile Gly Ser Gly Ala Gly Leu Ala Asp
 485 490 495
 Gly Ala Asp Leu Leu Gly Val Leu Pro Pro Gly Thr Tyr Pro Gly Asp
 500 505 510
 Phe Ser Arg Asp Thr His Phe Leu His Leu Arg Ser Ala Ser Leu Gly
 515 520 525
 30 Ser Gln His Leu Leu Gly Leu Pro Arg Asp Pro Ser Ser Val Ser
 530 535 540
 Gly Thr Phe Gly Cys Leu Gly Gly Arg Leu Thr Ile Pro Gly Thr Gly
 545 550 555 560
 35 Val Ser Leu Leu Val Pro Asn Gly Ala Ile Pro Gln Gly Lys Phe Tyr
 565 570 575
 Asp Leu Tyr Leu Arg Ile Asn Lys Thr Glu Ser Thr Leu Pro Leu Ser
 580 585 590
 Glu Gly Ser Gln Thr Val Leu Ser Pro Ser Val Thr Cys Gly Pro Thr
 595 600 605
 40 Gly Leu Leu Leu Cys Arg Pro Val Val Leu Thr Val Pro His Cys Ala
 610 615 620
 Glu Val Ile Ala Gly Asp Trp Ile Phe Gln Leu Lys Thr Gln Ala His
 625 630 635 640
 Gln Gly His Trp Glu Glu Val Val Thr Leu Asp Glu Glu Thr Leu Asn

	645	650	655
	Thr Pro Cys Tyr Cys Gln Leu Glu Ala Lys Ser Cys His Ile Leu Leu		
	660	665	670
	Asp Gln Leu Gly Thr Tyr Val Phe Thr Gly Glu Ser Tyr Ser Arg Ser		
	675	680	685
5	Ala Val Lys Arg Leu Gln Leu Ala Ile Phe Ala Pro Ala Leu Cys Thr		
	690	695	700
	Ser Leu Glu Tyr Ser Leu Arg Val Tyr Cys Leu Glu Asp Thr Pro Ala		
	705	710	715
	720 Ala Leu Lys Glu Val Leu Glu Leu Glu Arg Thr Leu Gly Gly Tyr Leu		
10	725	730	735
	Val Glu Glu Pro Lys Thr Leu Leu Phe Lys Asp Ser Tyr His Asn Leu		
	740	745	750
	Arg Leu Ser Leu His Asp Ile Pro His Ala His Trp Arg Ser Lys Leu		
	755	760	765
15	Leu Ala Lys Tyr Gln Glu Ile Pro Phe Tyr His Val Trp Asn Gly Ser		
	770	775	780
	Gln Lys Ala Leu His Cys Thr Phe Thr Leu Glu Arg His Ser Leu Ala		
	785	790	795
	800 Ser Thr Glu Phe Thr Cys Lys Val Cys Val Arg Gln Val Glu Gly Glu		
20	805	810	815
	Gly Gln Ile Phe Gln Leu His Thr Thr Leu Ala Glu Thr Pro Ala Gly		
	820	825	830
	Ser Leu Asp Ala Leu Cys Ser Ala Pro Gly Asn Ala Ala Thr Thr Gln		
	835	840	845
25	Leu Gly Pro Tyr Ala Phe Lys Ile Pro Leu Ser Ile Arg Gln Lys Ile		
	850	855	860
	Cys Asn Ser Leu Asp Ala Pro Asn Ser Arg Gly Asn Asp Trp Arg Leu		
	865	870	875
	880 Leu Ala Gln Lys Leu Ser Met Asp Arg Tyr Leu Asn Tyr Phe Ala Thr		
30	885	890	895
	Lys Ala Ser Pro Thr Gly Val Ile Leu Asp Leu Trp Glu Ala Arg Gln		
	900	905	910
	Gln Asp Asp Gly Asp Leu Asn Ser Leu Ala Ser Ala Leu Glu Glu Met		
	915	920	925
35	Gly Lys Ser Glu Met Leu Val Ala Met Thr Thr Asp Gly Asp Cys		
	930	935	940

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:

40 (A) LENGTH: 102 amino acids
 (B) TYPE: amino acid
 (C) STRANDEDNESS: not relevant
 (D) TOPOLOGY: not relevant

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

Asp Glu Glu Thr Leu Asn Thr Pro Cys Tyr Xaa Gln Leu Glu Pro Arg
1 5 10 15
Ala Cys Xaa Ile Leu Leu Asp Gln Leu Gly Thr Tyr Val Phe Thr Gly
20 25 30
5 Glu Ser Tyr Ser Arg Ser Ala Val Lys Arg Leu Gln Leu Ala Val Phe
35 40 45
Ala Pro Ala Leu Cys Thr Ser Leu Glu Tyr Ser Leu Arg Val Tyr Cys
50 55 60
10 Leu Glu Asp Thr Pro Val Ala Leu Lys Glu Val Leu Glu Leu Glu Arg
65 70 75 80
Thr Leu Gly Gly Tyr Leu Val Glu Glu Pro Lys Pro Leu Met Phe Lys
85 90 95
Asp Ser Tyr His Asn Leu
100

15

WHAT IS CLAIMED IS:

1. An isolated vertebrate UNC-5 protein comprising SEQ ID NO: 5, 6, 7 or, 8, or a fragment thereof having vertebrate UNC-5-specific activity.

5 2. An isolated protein according to claim 1, wherein said protein specifically binds a natural netrin protein.

3. A recombinant nucleic acid encoding a protein according to claim 1.

4. A cell comprising a nucleic acid according to claim 3.

10 5. A method of making an isolated vertebrate UNC-5 protein, comprising steps: introducing a nucleic acid according to claim 3 into a host cell or cellular extract, incubating said host cell or extract under conditions whereby said nucleic acid is expressed as a transcript and said transcript is expressed as a translation product comprising said protein, and isolating said 15 translation product.

6. An isolated vertebrate UNC-5 protein made by the method of claim 5.

20 7. An isolated vertebrate *unc-5* nucleic acid comprising SEQ ID NO: 1, 2, 3, or 4, or a fragment thereof having at least 24 consecutive bases of SEQ ID NO: 1, 2, 3, or 4 and sufficient to specifically hybridize with a nucleic acid having the sequence of the corresponding SEQ ID NO: 1, 2, 3, or 4 in the presence of natural *C. elegans unc-5* cDNA.

25 8. A method of screening for an agent which modulates the binding of a vertebrate UNC-5 protein to a binding target, said method comprising the steps of:

incubating a mixture comprising:

an isolated protein according to claim 1,

a binding target of said protein, and

a candidate agent;

30 under conditions whereby, but for the presence of said agent, said protein specifically binds said binding target at a reference affinity;

detecting the binding affinity of said protein to said binding target to determine an agent-biased affinity,

wherein a difference between the agent-biased affinity and the reference affinity indicates that said agent modulates the binding of said protein to said binding target.

5 9. A method according to claim 8, wherein said binding target is a natural netrin protein.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/03143

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : C07K 1/00, 14/00, 17/00; C07H 21/02, 21/04; G01N 33/53
US CL : 530/350; 536/23.1; 435/7.1

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 530/350; 536/23.1; 435/7.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

DIALOG: DATABASES WPI, MEDLINE, USPATFUL. AUTHOR AND WORD. SEARCH TERMS INCLUDE UNC-5 AND VERTEBRATE.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Database Medline on Dialog, US National Library fo Medicine, (Bethesda, MD, USA), No. 08202090 95037661, CULOTTI JG. 'Axon Guidance mechanisms in Caenorhabditis elegans,' Current opinion in Genetics and Development, abstract, August 1994, Vol. 4, No. 4, pages 587-595, see entire document.	1-9

 Further documents are listed in the continuation of Box C. See patent family annex.

* Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A" document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E" earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O" document referring to an oral disclosure, use, exhibition or other means		
"P" document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search	Date of mailing of the international search report
06 APRIL 1998	09 JUN 1998
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	Authorized officer HEATHER BAKALYAR
Facsimile No. (703) 305-3230	Telephone No. (703) 308-0196